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Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions

Jean-Christophe Palauqui, Taline Elmayan, Jean-Marie Pollien and Hervé Vaucheret[1]

Laboratoire de Biologie Cellulaire, INRA, 78026 Versailles Cedex, France

[1]Corresponding author

Using grafting procedures, we investigated the transmission of co-suppression of nitrate reductase and nitrite reductase host genes and transgenes and of post-transcriptional silencing of a *uidA* transgene encoding glucuronidase in tobacco. We demonstrate that silencing is transmitted with 100% efficiency from silenced stocks to non-silenced scions expressing the corresponding transgene. Transmission is unidirectional from stock to scion, transgene specific, locus independent and requires the presence of a transcriptionally active transgene in the target scion. The transmission of co-suppression occurs when silenced stocks and non-silenced target scions are physically separated by up to 30 cm of stem of a non-target wild-type plant. Taken together, these results suggest that a non-metabolic, transgene-specific, diffusable messenger mediates the propagation of *de novo* post-transcriptional silencing through the plant.

Keywords: grafting/post-transcriptional gene silencing/ transgenic plants

Introduction

Introduction of a transgene encoding part or the entire coding sequence of a host gene can lead to co-suppression of the transgene and of all homologous host gene copies (Napoli *et al.*, 1990; Smith *et al.*, 1990; Van der Krol *et al.*, 1990; Hart *et al.*, 1992; Dorlhac de Borne *et al.*, 1994; de Carvalho-Niebel *et al.*, 1995; Vaucheret *et al.*, 1995). This phenomenon results in a strong reduction of both host gene and transgene steady-state mRNA levels, although the (trans)genes are transcribed at apparently normal rates in the nucleus, indicating that co-suppression is post-transcriptional (van Blockland *et al.*, 1994; de Carvalho-Niebel *et al.*, 1995; Kunz *et al.*, 1996; Vaucheret *et al.*, 1997). Post-transcriptional silencing can also affect foreign transgenes that are not homologous to host genes (Dehio and Schell, 1994; Ingelbrecht *et al.*, 1994; Elmayan and Vaucheret, 1996). When the transgenes express a cDNA derived from part of the genome of an RNA virus, plants showing post-transcriptional silencing of the transgene display resistance against the corresponding virus, i.e. accumulation of the virus in the cytoplasm is strongly reduced (Lindbo *et al.*, 1993; Smith *et al.*, 1994; Mueller *et al.*, 1995; English *et al.*, 1996; Goodwin *et al.*, 1996; Sijen *et al.*, 1996).

Although numerous systems of post-transcriptional silencing have been studied, little is known about the actual mechanism by which it takes place. A 'biochemical switch' model suggests that when a transgene is expressed under the control of a strong promoter, the level of RNA can reach a 'threshold' level that triggers specific degradation of all homologous RNA in the cytoplasm (Meins, 1989; Dehio and Schell, 1994; Meins and Kunz, 1994; Dougherty and Parks, 1995; Elmayan and Vaucheret, 1996). Different observations are consistent with the hypothesis of RNA dosage: (i) transgenes expressed under the control of a 35S promoter with a double enhancer are much more often silenced than transgenes expressed under the control of wild-type 35S (Elmayan and Vaucheret, 1996; Jorgensen *et al.*, 1996); (ii) homozygous plants are much more often silenced than hemizygous plants (de Carvalho *et al.*, 1992; Hart *et al.*, 1992; Dehio and Schell, 1994; Dorlhac de Borne *et al.*, 1994; Palauqui and Vaucheret, 1995; Elmayan and Vaucheret, 1996); (iii) silencing can occur in haploid or hemizygous plants carrying a single copy of the transgene, suggesting that DNA–DNA interactions between either allelic or ectopic copies are not required (Elmayan and Vaucheret, 1996); (iv) post-transcriptional co-suppression of homologous host genes is inhibited when the transgene is inactivated at the transcriptional level (Vaucheret *et al.*, 1997); (v) infection of non-silenced transgenic plants by a homologous RNA virus can lead to post-transcriptional silencing of the transgene and subsequently to virus resistance, a phenomenon called 'recovery' (Lindbo *et al.*, 1993).

However, striking data indicate that weakly transcribed or promoterless (untranscribed) transgenes can trigger post-transcriptional silencing of homologous host genes (van Blockland *et al.*, 1994). In addition, transgene-mediated RNA virus resistance does not always correlate with transgene expression level (Mueller *et al.*, 1995; English *et al.*, 1996). In these cases, silencing is assumed to result from changes occurring at the DNA level. Indeed, in such transgenic lines gene silencing is correlated with the presence of inverted repeats of the T-DNA, suggesting that ectopic pairing between transgene copies or between transgenes and homologous host genes can lead to the synthesis of unproductive (aberrant) RNA that triggers specific degradation of all homologous RNA in the cytoplasm (van Blockland *et al.*, 1994; Baulcombe and English, 1996; English *et al.*, 1996). Since not all the data can be explained with a single model, there might be (at least) two alternative ways to trigger the same effect. However, these two ways may not be mutually exclusive, since those transgenic plants showing silencing of a foreign transgene with very high efficiency (Elmayan and Vaucheret, 1996) or showing the best resistance to virus infection (Sijen *et al.*, 1996) exhibit both a high level of transcription and transgene repeats.

The actual mechanism leading to RNA degradation therefore remains unclear at the cell level. At the plant level, things are no clearer. Several studies have reported that co-suppression is non-clonal, as opposed to transcriptional silencing, which resembles position effect variegation (PEV) in *Drosophila* (Ye and Signer, 1996) and which is assumed to be clonal, like PEV (Karpen, 1994). Indeed, particular non-clonal spatial patterns of co-suppression have been described in the case of chalcone synthase (Napoli *et al.*, 1990; van der Krol *et al.*, 1990; van Blockland *et al.*, 1994; Jorgensen, 1995; Jorgensen *et al.*, 1996), chitinase (Hart *et al.*, 1992; Kunz *et al.*, 1996), SAM synthase (Boerjan *et al.*, 1994), nitrate reductase and nitrite reductase (Palauqui *et al.*, 1996). In particular, we previously reported some striking and reproducible features in the evolution of nitrate reductase and nitrite reductase co-suppression spatial patterns during plant development which suggest the propagation of a silencing message through the plant (Palauqui *et al.*, 1996).

To elucidate the mechanism by which co-suppression takes place in the whole plant, we developed an artificial and efficient system based on grafting procedures. We demonstrate that nitrate reductase and nitrite reductase co-suppression and also post-transcriptional silencing of a foreign 70-*UidA* transgene encoding glucuronidase (Elmayan and Vaucheret, 1996) can be transmitted from silenced stocks to non-silenced scions. Using reciprocal grafts and sandwich grafts, we came to the conclusion that the transmission of post-transcriptional silencing is systemic, non-metabolic and transgene specific.

Results

Co-suppression of nitrate reductase host genes and transgenes is transmitted from silenced stocks to isogenic non-silenced scions

We reported previously that transgenic tobacco lines homozygous for a 35S-*Nia2* transgene can exhibit co-suppression of *Nia* host genes and transgenes, leading to dramatic and visible chlorosis (Dorlhac de Borne *et al.*, 1994; Palauqui and Vaucheret, 1995). Co-suppression occurs in each generation and affects a constant fraction of the progeny of these homozygous lines. The percentage of plants affected by co-suppression differs from one line to another, ranging from 3 to 57% (see Materials and methods). To test if co-suppression of the fraction of non-silenced (NS) plants could be triggered *de novo*, we grafted their upper part (NS scions) onto the lower part of silenced (S) isogenic plants (S stocks). As controls, we kept the lower part of the NS plants growing (beheaded NS stocks) to check whether or not spontaneous triggering of co-suppression could have occurred. Figure 1 shows the steps of this grafting experiment. During the first week after grafting the scions, being deprived of their roots, wilted a little, but then recovered and by the third week began to grow well. Chlorosis appeared uniformly on all the developing leaves of the grafted scions, suggesting that co-suppression had occurred. Conversely, the new leaves which appeared on the corresponding beheaded NS stocks remained green. RNA was extracted from leaves of the grafted scions and from the corresponding beheaded stocks and hybridized with a *Nia2* probe. RNA gel blot analysis (Figure 2A) confirmed that the chlorosis of grafted

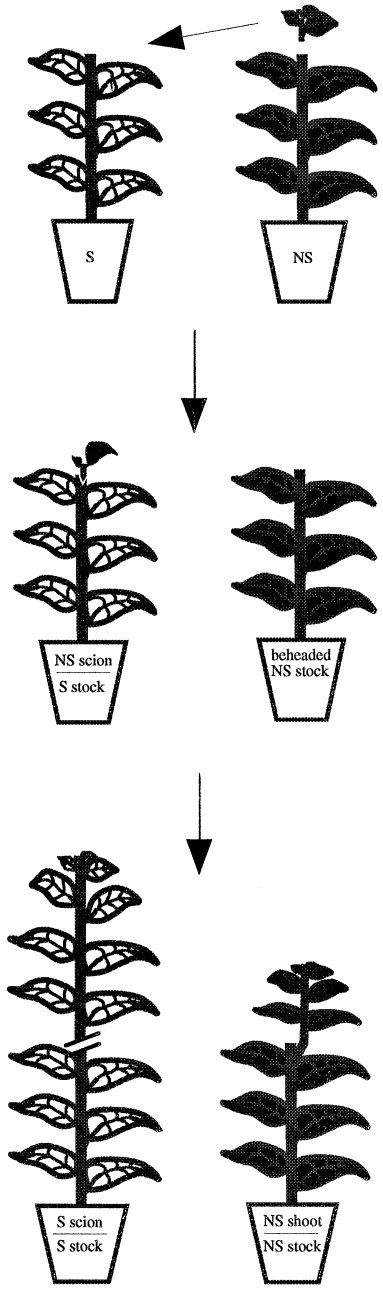

Fig. 1. Schematic representation of grafting transmission. The terminal apex of a NS plant (NS scion, represented in black) is grafted onto a beheaded S plant (S stock, represented in white). Silencing of the grafted NS scion is monitored by the appearance of chlorosis (in the case of nitrate reductase and nitrite reductase) or by fluorimetric dosage (in the case of glucuronidase) and confirmed by RNA gel blot analysis. To verify that silencing of the scion does not result from spontaneous triggering of the plants from which they are taken, the lower part of the plant (beheaded NS stock) is kept to allow development of lateral shoots.

scions was due to *de novo* co-suppression and not to spontaneous triggering, since no *Nia* mRNA was detected in the grafted scions (lane 7), whereas shoots of the corresponding beheaded NS stocks accumulated *Nia* mRNA normally (lane 1). The transmission of chlorosis (and co-suppression) was observed using seven independent transgenic lines (Table I, experiments 1–7) suggesting that *de novo* co-suppression of NS scions is

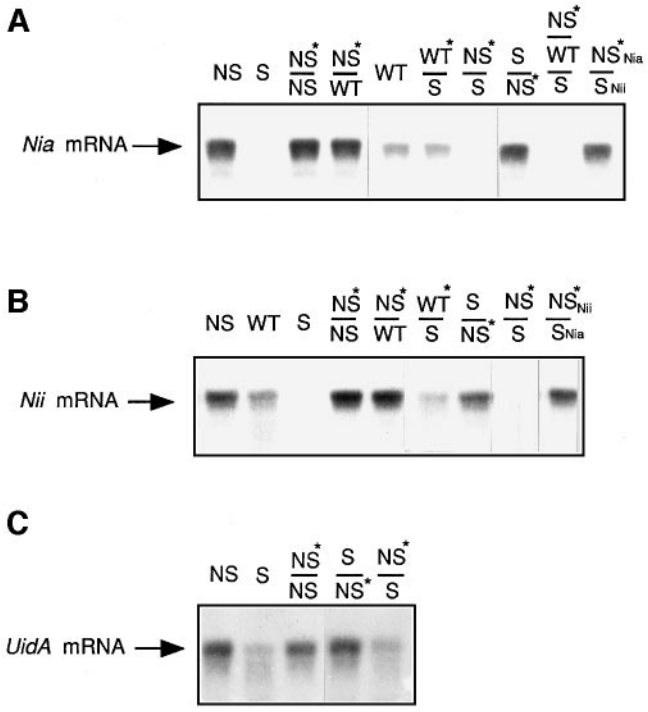

Fig. 2. Analysis of steady-state RNA levels in grafted plants. Total RNA was extracted from the leaves of either stocks or scions. Ten micrograms of RNA from each sample were probed. *The part (stock or scion) of the graft that was analysed: NS, non-silenced; S, silenced; WT, wild-type. (**A**) Analysis of *Nia* steady-state level in grafted plants. (**B**) Analysis of *Nii* steady-state level in grafted plants. (**C**) Analysis of *uidA* steady-state level in grafted plants.

triggered with 100% efficiency by grafting onto isogenic S stocks.

Transmission is locus independent

The target NS scions described above were grafted onto non-isogenic S stocks carrying the same 35S-*Nia2* transgene inserted at a different position within the genome. As described before, beheaded NS plants used as a reservoir for scions were followed for their ability to trigger natural co-suppression. Transmission of co-suppression from S stocks to NS scions was 100% efficient, irrespective of the transgene locus analysed, i.e. irrespective of T-DNA copy number, transgene locus structure and genomic position (Table I, experiments 8–14), thus indicating that transmission is locus independent.

Co-suppression is not due to the grafting procedure or to grafting onto chlorotic plants

In order to ensure that the grafting procedure was not involved in the triggering of co-suppression, transgenic NS target scions were grafted onto isogenic or non-isogenic NS stocks or wild-type (WT) stocks. No chlorosis appeared in these target scions (Table II, experiments 1 and 2). RNA gel blot analysis confirmed the absence of co-suppression (Figure 2A, lanes 3 and 4), indicating clearly that the grafting procedure was not responsible for the triggering of *de novo* co-suppression.

We also confirmed that the triggering of co-suppression was not due to grafting onto nitrate reductase-deficient chlorotic stocks. Transgenic NS target scions were grafted onto the non-transgenic chlorotic tobacco mutant NIA30,

Table I. Transmission of nitrate reductase co-suppression from stock to scion

Experiment	NS scion	S stock	No. of grafts	No. of silenced scions
1	27-44.7	27-44.7	2	2
2	30-18.1	30-18.1	2	2
3	30-18.2	30-18.2	4	4
4	30-46.7	30-46.7	42	42
5	30-51.7	30-51.7	1	1
6	30-91.3	30-91.3	20	20
7	34-2.5	34-2.5	10	10
8	27-44.7	30-18.9	5	5
9	30-46.7	27-44.7	10	10
10	30-46.7	30-18.9	50	50
11	30-46.7	30-91.3	20	20
12	30-51.7	30-18.9	1	1
13	30-91.3	30-18.9	30	30
14	30-91.3	30-46.7	20	20

NS scions were grafted onto isogenic (experiments 1–7) or non-isogenic (experiments 8–14) S stocks carrying the same 35S-*Nia2* transgene.

which has defective *Nia1* and *Nia2* genes and does not produce any functional nitrate reductase protein (Müller, 1983). None of the target scions became chlorotic (Table II, experiment 4), thus indicating that the presence of a silenced 35S-*Nia2* transgene in the stocks is required and that *de novo* co-suppression is not a metabolic effect due to grafting onto a chlorotic nitrate reductase-deficient stock.

Transmission requires a competent scion

To test if the endogenous *Nia* genes can be silenced in the absence of 35S-*Nia2* transgenes, WT scions were grafted onto transgenic S scions. None of the WT scions became silenced (Table III). No difference in RNA steady-state level was observed between WT plants and WT scions grafted on S stocks (Figure 2A, lanes 5 and 6). This result suggests that the presence of a 35S-*Nia2* transgene is required for triggering.

To test if transgene transcription was required for triggering of silencing by grafting, we introduced the super-silencing locus 271 into the NS target scions.The 271 locus inactivates any transgene driven by the 35S promoter at the transcriptional level (Vaucheret, 1993; Elmayan and Vaucheret, 1996; Park *et al.*, 1996). As NS target scions we used either hybrids between the transgenic line 30-18.9 (see Materials and methods) and the transgenic line 271-22-2.11 (see Materials and methods) or hybrids between line 30-18.9 and a WT plant. Under natural conditions of growth 15% of the hybrids between 30-18.9 and WT were affected by co-suppression, while hybrids between 30-18.9 and 271-22-2.11 were not (Vaucheret *et al.*, 1997). When grafted onto S scions, NS hybrid scions between 30-18.9 and WT became silenced, while NS hybrid scions between 30-18.9 and 271-22-2.11 did not (Table III). This result indicates that the presence of the 35S-*Nia2* transgene in the scion is not sufficient *per se* and that the transgene must be transcriptionally active for the scion to be competent for silencing.

Transmission is unidirectional

The direction of transmission was investigated by reciprocal grafts: NS and S plants were used either as stocks or

Table II. Transmission of *Nia*, *Nii* and *UidA* silencing from stock to scion

Experiment	Scion	State[a]	Stock	State	No. of grafts	No. of silenced scions
1	35S-Nia2	NS	PBD6	WT	30	0
2	35S-Nia2	NS	35S-Nia2	NS	30	0
3	35S-Nia2	NS	35S-Nia2	S	217	217
4	35S-Nia2	NS	NIA30	NR mutant	15	0
5	35S-Nia2	NS	70-Nii1	S	10	10
6	35S-Nia2	NS	70-UidA	S	15	0
7	70-Nii1	NS	PBD6	WT	10	0
8	70-Nii1	NS	70-Nii1	NS	10	0
9	70-Nii1	NS	70-Nii1	S	10	10
10	70-Nii1	NS	35S-Nii1	S	20	20
11	70-Nii1	NS	35S-Nia2	S	15	0
12	70-UidA	NS	PBD6	WT	12	0
13	70-UidA	NS	70-UidA	NS	12	0
14	70-UidA	NS	70-UidA	S	48	48
15	70-UidA	NS	35S-Nia2	S	10	0

[a]NS, non-silenced; WT, wild-type; S, silenced.
NS scions were grafted onto various types of stocks: experiments 1, 7 and 12, onto wild-type stocks; experiments 2, 8 and 13, onto NS stocks carrying the same transgene; experiments 3, 9, 10 and 14, onto S stocks silenced for the same transgene; experiment 4, onto a non-transgenic mutant phenocopying silenced plants; experiments 5, 6, 11 and 15, onto S stocks silenced for a different transgene.

scions. The apices of the two types of plants were grafted reciprocally onto the beheaded stock. NS scions grafted onto S stocks became chlorotic, whereas NS stocks onto which S scions were grafted did not (data not shown). Lateral buds developing from NS stocks carrying S scions were not chlorotic, suggesting that transmission of co-suppression was unidirectional from stock to scion. RNA gel blot analysis confirmed the absence of *de novo* co-suppression in leaves of NS stocks onto which S scions were grafted (Figure 2A, lane 8).

Transmission does not require the roots of S stocks

To test if the roots of S stocks were required for transmission from stocks to scions, we grafted the upper part of S plants onto WT stocks and one month later we grafted NS target scions onto these hybrid S/WT stocks. Co-suppression was triggered with 100% efficiency in these NS scions (data not shown), thus indicating that transmission can occur in the absence of the roots of S stocks. This result suggests that some information is produced by stems and/or leaves of S stocks and migrates to NS scions to trigger *de novo* co-suppression.

Transmission occurs through 30 cm of WT stem

To test if a direct contact between scions and stocks was required for triggering of *de novo* co-suppression, we inserted 10, 20 or 30 cm of stem of a WT plant between NS scions and S stocks. Co-suppression was transmitted in all cases, irrespective of the size of the inserted WT stem (Table IV). RNA gel blot analysis confirmed the triggering of co-suppression (Figure 2A, lane 9), thus indicating that the information which triggers *de novo* co-suppression can migrate a long distance.

Co-suppression of nitrite reductase host genes and transgenes is also transmitted from silenced stocks to non-silenced scions

Similar experiments were performed using transgenic plants carrying either the *Nii1* cDNA encoding nitrite reductase under the control of the 35S promoter (construct 35S-*Nii1*) or the *Nii* gene with its own regulatory sequences cloned downstream of the enhancer of the 35S promoter (construct 70-*Nii1*). The transgenic lines 461-2.1, 475-2.1 and 475-11.5, homozygous for one or the other construct and all showing co-suppression of nitrite reductase with 100% efficiency (Vaucheret *et al.*, 1995; Palauqui *et al.*, 1996), were used as chlorotic S stocks. Hybrids between line 461-2.1 and line 461-7.8, which trigger co-suppression with an efficiency of 2% (Vaucheret *et al.*, 1995), were used as target NS scions. Grafted NS scions became chlorotic in all cases (Table II, experiments 9 and 10) and RNA gel blot analysis confirmed that *de novo* co-suppression had occurred (Figure 2B, lane 8 versus lane 1). As found previously for nitrate reductase, *de novo* co-suppression was locus-independent, unidirectional, did not require the presence of the roots of the S stocks and was not due to the grafting procedure (Table II, experiments 7 and 8; see also Figure 2B, lanes 4, 5 and 7). In addition, expression of host *Nii* genes in WT scions grafted onto S stocks was not affected (Figure 2B, lanes 2 and 6), thus confirming that the presence of a transgene is required in the target scion.

Table III. Transgene transcription requirement in grafted scions

Scion	State[a]	Stock	State	No. of grafts	No. of silenced scions
PBD6	WT	30-18.9	S	12	0
WT×30-18.9	NS	30-18.9	S	12	12
271×30-18.9	NS	30-18.9	S	12	0

[a]NS, non-silenced; WT, wild-type; S, silenced.
Various types of scions were grafted onto S stocks silenced for the 35S-*Nia2* transgene: the WT line PBD6 does not carry any 35S-*Nia2* transgene locus; the hybrid WT×30-18.9 carries two transcriptionally active 35S-*Nia2* transgene loci; and the hybrid 271×30-18.9 carries two inactive 35S-*Nia2* transgene loci.

Transmission is (trans)gene specific

To test whether the ability of S stocks to trigger *de novo* co-suppression in NS scions was (trans)gene specific or

Table IV. Long distance transmission of *Nia* co-suppression

Scion	State[a]	Sandwich	Length (cm)	Stock	State	No. of grafts	No. of silenced scions
35S-Nia2	NS	WT	10	35S-Nia2	S	3	3
35S-Nia2	NS	WT	20	35S-Nia2	S	3	3
35S-Nia2	NS	WT	30	35S-Nia2	S	3	3

[a]NS, non-silenced; WT, wild-type; S, silenced.
Sandwich grafts were performed by intercalation of various lengths of the stem of a wild-type plant between NS scions and S stocks carrying a 35S-*Nia2* transgene.

not, reciprocal grafts were made using plants silenced for either nitrate or nitrite reductase. *Nia* NS scions were grafted onto *Nii* S stocks and *Nii* NS scions were grafted onto *Nia* S stocks. None of the scions became chlorotic (Table II, experiments 5 and 11). RNA gel blot analysis confirmed that co-suppression had not occurred in the scions (Figure 2A and B, lanes 10 and 9 respectively). These results suggest that the silencing message which migrates from S stocks to NS scions is (trans)gene specific, thus confirming that *de novo* co-suppression is not related to changes in nitrogen metabolism, as previously suggested by the absence of triggering in NS scions grafted onto nitrate reductase-deficient mutants.

Post-transcriptional silencing of a uidA transgene is also transmitted from stocks to scions

Post-transcriptional silencing of transgenes which do not have any homologues within the genome very much resembles co-suppression: it occurs in each generation during the development of the plants and results in the degradation of RNA which is still transcribed in the nucleus. Using transgenic tobacco plants silenced for the *uidA* gene encoding glucuronidase (Elmayan and Vaucheret, 1996), we tested if silencing can be transmitted from stocks to scions by grafting. As NS target scions we used hemizygous plants derived from lines 23b6 and 23b9 (homozygous plants derived from these lines are silenced, whereas hemizygous plants are not). As S stocks we used homozygous plants derived from lines 6b5 and 6b8 (homozygous and hemizygous plants derived from these lines are silenced). By fluorimetric measurements (data not shown) we found that in all cases NS scions grafted onto S stocks became silenced (Table II, experiment 14). Conversely, *uidA* NS scions grafted onto WT, *uidA* NS or *Nia* S stocks did not (Table II, experiments 12, 13 and 15). These results, confirmed by RNA gel blot analysis (Figure 2C), indicate that post-transcriptional silencing of *uidA* is transmissible by grafting, like co-suppression of *Nia* and *Nii* genes. In addition, it confirms that the message which is transmitted from stocks to scions and which mediates *de novo* transgene-specific silencing in grafted scions is not metabolic, since *uidA* is an exogenous transgene. Therefore, this message is assumed to be (in part) a transgene product.

Discussion

Transgene-specific post-transcriptional silencing can be transmitted by grafting from silenced transgenic tobacco plants to non-silenced plants expressing the corresponding transgene. Transmission was investigated using two systems of co-suppression, nitrate reductase (encoded by *Nia* genes) and nitrite reductase (encoded by *Nii* genes), and one system of post-transcriptional gene silencing, glucuronidase (encoded by the bacterial *uidA* gene). We previously reported some striking and reproducible features in the evolution of *Nia* and *Nii* co-suppression patterns during plant development which suggest propagation of a silencing message through the plant (Palauqui *et al.*, 1996). Co-suppression was shown to occur primarily in one leaf and then to propagate to the upper part of the plant in a transgene-specific manner. Nitrate reductase co-suppression appeared as interveinal spots or vein-localized areas on one leaf situated at the bottom of the plant and then propagated to the upper leaves, primarily those on the same side of the plant. Nitrite reductase co-suppression appeared as interveinal spots or vein-localized areas on one leaf situated in the middle of the plant and then propagated to all the upper leaves with increasing silencing efficiency, whereas the lower leaves remained unaffected, a pattern resembling that found in SAM synthase silencing (Boerjan *et al.*, 1994). Since these non-clonal patterns were observed reproducibly in all transgenic lines silenced for a given gene, we proposed that a transgene-specific message involved in the control of post-transcriptional silencing diffuses through the plant in a specific manner (Palauqui *et al.*, 1996).

Grafting experiments realized with our three transgenic silencing systems showed that non-silenced transgenic plants (NS scions) grafted onto the corresponding silenced plants (S stocks) become silenced with 100% efficiency (295 grafts). Control grafts performed onto WT or NS stocks indicated that grafting *per se* does not elicit co-suppression (174 grafts). Reciprocal grafts of S scions onto NS stocks did not result in triggering of co-suppression in the stocks, thus indicating that transmission is unidirectional from stock to scion, like the natural propagation of nitrate reductase and nitrite reductase co-suppression from the base to the top of the plant. This result suggests that a silencing message migrates from the base to the top of the plant and not from the top to the base, although it is known that stocks and scions can communicate in both directions. Viruses and photo-assimilates migrate preferentially from scions to stocks, but transport from stocks to scions has been occasionally reported in graft experiments (Kollmann and Glockmann, 1990; Matthews, 1991). The unidirectional transmission of silencing from stocks to scions suggests the existence of an ascending message for a phenomenon we call systemic acquired silencing (SAS), by analogy with the ascending message for systemic acquired resistance (SAR), which has already been characterized by grafting (Vernooij *et al.*, 1994).

Previous reports indicated that the upper part of transgenic plants expressing a fragment of a viral genome can sometimes become resistant to virus infection after inoculation and infection of the lower part of the plant (Lindbo *et al.*, 1993). To explain the 'recovery' phenotype observed in developing leaves, Baulcombe (1996) proposed that only the dividing cells were competent for silencing. In dividing cells the viral RNA could enter the nucleus, deprived transiently of its membrane, and interact with the transgene, thus allowing production of aberrant RNAs (aRNA) that trigger silencing. In the light of our results, the initial production of a SAS message in dividing cells and its upward propagation could actively contribute to the appearance of the 'recovery' phenotype in the developing leaves. In our experiments where S scions were grafted onto NS stocks, neither the leaves of the NS stocks (which do not contain dividing cells) nor the lateral buds which developed due to the loss of apical dominance (and which do contain dividing cells) showed co-suppression, suggesting either that dividing cells situated below the S scions did not receive the SAS message or that they were not competent to receive and/or to respond to the silencing message. Since various spatial patterns of co-suppression have been observed using different plant species and different transgenes under the control of the 35S promoter (Boerjan *et al.*, 1995; Jorgensen *et al.*, 1996; Kunz *et al.*, 1996; Palauqui *et al.*, 1996), it is possible that both the regulation of endogenous genes and the development of plant architecture could influence the mode of transmission of the SAS message. Therefore, although it is clear that the transmission of *Nia*, *Nii* and *uidA* silencing by grafting is unidirectional from the base to the top of transgenic tobacco plants, one cannot answer whether the SAS message is exclusively ascendant or not.

According to the concept of SAS we can distinguish two partners: the source (S stocks) and the target (NS scions). What are the requirements for these two partners to play their role? (i) The source must be a transgenic plant silenced post-transcriptionally for the (trans)gene present in the target NS scion. Grafting *Nia* NS scions onto *Nia*-deficient stocks mutated in the host *Nia* genes, instead of *Nia*-deficient stocks silenced post-transcriptionally, did not trigger silencing. Similarly, grafting of *Nia* NS scions onto *Nii* S stocks did not trigger silencing, indicating that silencing of *Nia* is not related to a deficiency in the nitrogen metabolism of the stocks. (ii) The source does not need to grow on its own roots. Grafting of S plants onto WT plants and subsequent grafting of NS scions onto these S/WT stocks triggered silencing in the scions. This result indicates that the silencing message emitted by the source is not produced in the roots and thus is probably produced by the leaves or the stem. (iii) The target must carry the same transgene as the source. *Nia* NS scions grafted onto *Nii* S stocks or *Nii* NS scions grafted onto *Nia* S stocks did not become silenced. Similar results were obtained with *uidA* NS scions grafted onto *Nia* S stocks or *Nia* NS scions grafted onto *uidA* S stocks. These results indicate that the message emitted by the source is transgene specific. The position, copy number and arrangement of the transgene within the genome does not seem to play any role. (iv) The target must carry a transgene in a transcriptionally active state. WT scions grafted onto S scions did not show silencing of *Nia* or *Nii* host genes, indicating that WT plants are not competent for silencing. NS scions carrying a 35S-*Nia2* transgene which is transcriptionally silenced by a 35S-specific silencing locus that does not affect expression of the target *Nia* host genes (Vaucheret, 1993; Elmayan and Vaucheret, 1996; Thierry and Vaucheret, 1996) cannot be silenced by grafting onto *Nia* S stocks, thus indicating that the presence of the transgene *per se* is insufficient to make a competent target. (v) Sources and targets do not need to be directly in contact. The presence of up to 30 cm of stem of a WT plant (that does not trigger silencing) intercalated between S stocks and NS scions still allows triggering of silencing, thus indicating that the SAS message migrates over long distances, like the SAR message (Vernooij *et al.*, 1994).

What is the nature of the SAS message? Meins and Kunz (1994) proposed a heuristic positive autoregulation model in which a diffusible factor activates chitinase host gene and transgene expression by interacting with coding sequence-specific regulatory proteins to alter the rate at which complete, functional transcripts are produced. Jorgensen (1995) proposed a prepattern threshold hypothesis to account for the three-dimensional organization of chalcone synthase (*chs*) co-suppression patterns in petunia corolla. According to this hypothesis, transcription factors or their effector molecules could locally influence *chs* transcription rate, leading to a threshold concentration of nuclear transcript that shifts the cell from the non-silent to the silent state. Meanwhile, the analysis of transgenic petunia plants silenced for *chs* host genes and transgenes revealed the presence of shorter poly(A)$^-$ *chs* RNAs (Metzlaff *et al.*, 1997). The authors proposed an autoregulatory degradative model in which silencing occurs by means of pairing–cleavage cycles between shorter (aberrant) poly(A)$^-$ *chs* RNAs and complete, functional *chs* mRNAs, as a result of internal sequence complementarity. In *Neurospora crassa* a transgene-mediated post-transcriptional silencing phenomenon which resembles co-suppression in plants has been described and referred to as quelling (Cogoni *et al.*, 1996). In this case it was shown that spontaneous or forced dikaryons containing both quelled transgenic nuclei and non-quelled WT nuclei rapidly exhibit in most cases a unique quelled phenotype, suggesting that quelling involves the production of a *trans*-acting effector by quelled nuclei that acts as a dominant trait. In addition, it suggests that this process is not nucleus limited. This *trans*-acting effector was proposed to involve a transgene product, probably RNA. In transgenic tobacco plants the SAS message involved in the transmission of *Nia*, *Nii* and *uidA* silencing is transgene specific, although these three transgenes are driven by the 35S promoter. This result excludes a possible diffusion of 35S-specific transcription factors that could elevate the target gene RNA level over a threshold level that triggers silencing. In addition, SAS can affect the *uidA* gene, which has no homologues within the genome. This suggests that the messenger that mediates SAS consists (at least in part) of a transgene product, probably aberant poly(A)$^-$ RNAs, as suggested in the case of *chs* co-suppression (Metzlaff *et al.*, 1997).

How does the SAS message get through the plant? In compatible heterografts stocks and scions belonging to different species or genera form successful unions based

upon vascular connections linking the conducting tissues. Ultrastructural differences between stock and scion allow the identification of each cell in the graft union. In this way, interconnections of parenchyma cells by plasmodesmata were shown to be secondarily formed in a non-division wall (Kollman and Glockmann, 1990). In autografts the mechanisms are poorly understood, since species-specific ultrastructural differences are lacking. However, vascular connections probably involve cellular remodelling that takes place early in the formation of the graft union. The SAS signal could travel via plasmodesmata and/or vascular connections. The fact that macromolecules travel through the plant is not a new assumption, since many plant viruses use such a system to invade new areas of the plants during infection. More recently, host macromolecules were identified that are transported in plants, namely the KNOTTED transcription factors. In this case, KNOTTED proteins and probably corresponding mRNAs are able to move from cells where they are synthesized to cells where they are lacking, demonstrating that cell-to-cell post-transcriptional regulation can occur in plants (Lucas *et al.*, 1995). At the present time it is not known whether the SAS messenger consists solely of transgene-encoded RNA or if it involves a host helper molecule, as for virus transmission. It is also not known whether the SAS messenger travels only from cell to cell via plasmodesmata or if it can migrate over long distances through vascular connections. The identification of mutants impaired in gene silencing and isolation of the corresponding genes will help to define which components of the cell are involved in the propagation of post-transcriptional silencing.

Materials and methods

Plant material

The transgenic tobacco lines 27-44.7, 30-18.1, 30-18.2, 30-46.7, 30-51.7, 30-91.3 and 34-2.5 are homozygous for one 35S-*Nia2* transgene locus, while the transgenic line 30-18.9 is homozygous for the two loci carried by lines 30-18.1 and 30-18.2 (Dorlhac de Borne *et al.*, 1994; Palauqui and Vaucheret, 1995). In each generation only a fraction of plants derived from these lines is affected by co-suppression. The frequencies of nitrate reductase co-suppression are respectively 42, 15, 12, 10, 3, 8, 57 and 100%.

The transgenic tobacco line 461-2.1 is homozygous for one 70-*Nii1* transgene locus (Vaucheret *et al.*, 1995). The transgenic lines 475-2.1 and 475-11.5 are homozygous for one 35S-*Nii1* transgene locus (Palauqui *et al.*, 1996). The frequencies of nitrite reductase co-suppression of these lines are respectively 100, 100 and 97%. An F1 hybrid between 461-2.1 and 461-7.8 was also used, with a frequency of co-suppression of 2% (Vaucheret *et al.*, 1995).

The transgenic tobacco lines 6b5, 6b8, 23b6 and 23b9 are homozygous for one 70-*UidA* transgene locus (Elmayan and Vaucheret, 1996). Lines 6b5 and 6b8 show 100% glucuronidase silencing when the transgene locus is in the homozygous or hemizygous state. Lines 23b6 and 23b9 show 100% glucuronidase silencing when homozygous for the transgene locus and 0% when hemizygous.

The transgenic tobacco line 271-22-2.11 is homozygous for two transgene loci. One transgene locus triggers both transcriptional silencing of any 35S-driven transgene and post-transcriptional silencing of nitrite reductase host genes (Vaucheret, 1993; Elmayan and Vaucheret, 1996; Park *et al.*, 1996). The other transgene locus carries a bean nitrite reductase gene that restores nitrite reductase activity (Thierry and Vaucheret, 1996).

Wild-type tobacco plants (cv Paraguay PBD6) and the NR-deficient tobacco mutant NIA30 (Müller, 1983) were used as grafting controls. Wild-type tobacco plants (cv Xanthi XHFD8) were used for sandwich grafts.

Graft techniques

Seeds of wild-type and transgenic plants were sown in a greenhouse and grown with a 15 h light/9 h dark photoperiod. Plants were grown 2–3 months before grafting.

Two distinct grafting methods were used. (i) In the cleft grafting method, the stock was beheaded 30–40 cm above the soil. The outermost cortex of the stock stem was cut longitudinally, thus giving rise to a cortex flap. The terminal apex of the plant used as scion was excised, bevelled and fastened to the stock between the flap and the main part of the stem using Parafilm. During the first week after grafting the scion was covered with an inverted transparent plastic tube and sealed with Parafilm in order to avoid dehydration. (ii) The other method consisted of diagonally beheading the scions and stocks 30–40 cm above the soil. Scions were directly fastened to the stocks using Parafilm.

Double sandwich grafts were performed using a combination of these two methods. Scions were first grafted onto WT plants using the cleft grafting method. Two weeks later these grafted plants were cut 10, 20 or 30 cm below the position of the first graft to serve as scions and were grafted onto stocks using the diagonal method.

Plant analysis

Silencing was monitored by the appearance of leaf chlorosis (in the case of nitrate reductase and nitrite reductase; Palauqui *et al.*, 1996) or by fluorimetric dosage (in the case of glucuronidase; Elmayan and Vaucheret, 1996). If sponteanous silencing of the beheaded NS plant used as a reservoir of NS scions occurred, the experiment was not taken into account. Silencing was confirmed by RNA gel blot analysis using *Nia2*, *Nii1* or *uidA* probes as described previously (Elmayan and Vaucheret, 1996; Palauqui *et al.*, 1996).

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References

Baulcombe,D.C. (1996) RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. *Plant Mol. Biol.*, **32**, 79–88.

Baulcombe,D.C. and English,J.J. (1996) Ectopic pairing of homologous DNA and post-transcriptional gene silencing in transgenic plants. *Curr. Opin. Biotechnol.*, **7**, 173–180.

Boerjan,W., Bauw,G., Van Montagu,M. and Inze,D. (1994) Distinct phenotypes generated by overexpression and suppression of *S*-adenosyl-L-methionine synthetase reveal developmental patterns of gene silencing in tobacco. *Plant Cell*, **6**, 1401–1414.

Cogoni,C., Irelan,J.T., Schumacher,M., Schmidhauser,T.J., Selker,E.U. and Macino,G. (1996) Transgene silencing of the *al-1* gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA–DNA interactions or DNA methylation. *EMBO J.*, **15**, 3153–3163.

de Carvalho,F., Gheysen,G., Kushnir,S., Van Montagu,M., Inzé,D. and Castresana,C. (1992) Suppression of β-1,3-glucanase transgene expression in homozygous plants. *EMBO J.*, **11**, 2595–2602.

de Carvalho-Niebel,F., Frendo,P., Van Montagu,M. and Cornelissen,M. (1995) Post-transcriptional co-suppression of beta-1,3-glucanase genes does not affect accumulation of transgene nuclear mRNA. *Plant Cell*, **7**, 347–358.

Dehio,C. and Schell,J. (1994) Identification of plant genetic loci involved in a posttranscriptional mechanism for meiotically reversible transgene silencing. *Proc. Natl Acad. Sci. USA*, **91**, 5538–5542.

Dorlhac de Borne,F., Vincentz,M., Chupeau,Y. and Vaucheret,H. (1994) Co-suppression of nitrate reductase host genes and transgenes in transgenic tobacco plants. *Mol. Gen. Genet.*, **243**, 613–621.

Dougherty,W.G. and Parks,T.D. (1995) Transgenes and gene suppression: telling us something new? *Curr. Opin. Cell Biol.*, **7**, 399–405.

Elmayan,T. and Vaucheret,H. (1996) Single copies of a strongly-expressed 35S-driven transgene undergo post-transcriptional silencing. *Plant J.*, **9**, 787–797.

English,J.J., Mueller,E. and Baulcombe,D.C. (1996) Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. *Plant Cell*, **8**, 179–188.

Goodwin,J., Chapman,K., Swaney,S., Parks,T.D., Wernsman,E.A. and Dougherty,W.G. (1996) Genetic and biochemical dissection of transgenic RNA-mediated virus resistance. *Plant Cell*, **8**, 95–105.

Hart,C.M., Fisher,B., Neuhaus,J.-M. and Meins,F. (1992) Regulated inactivation of homologous gene expression in transgenic *Nicotiana sylvestris* plants containing a defense-related tobacco chitinase gene. *Mol. Gen. Genet*., **235**, 179–188.

Ingelbrecht,I., Van Houdt,H., Van Montagu,M. and Depicker,A. (1994) Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. *Proc. Natl Acad. Sci. USA*, **91**, 10502–10506.

Jorgensen,R.A. (1995) Cosuppression, flower color patterns and metastable gene expression states. *Science*, **268**, 686–691.

Jorgensen R.A., Cluster,P. D., English,J. J., Que,Q. and Napoli,C.A. (1996) Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. *Plant Mol. Biol*., **31**, 957–973.

Karpen,G.H. (1994) Position-effect variegation and the new biology of heterochromatin. *Curr. Opin. Genet. Dev.*, **4**, 281–291.

Kollmann,R. and Glockmann,C. (1990) Sieve elements of graft unions. In Behnke,H.-D. and Sjolund,R.D. (eds), *Sieve Elements*. Springer Verlag, Berlin, Germany, pp. 219–237.

Kunz,C., Schöb,H., Stam,M., Kooter,J.M. and Meins,F. (1996) Developmentally regulated silencing and reactivation of tobacco chitinase transgene expression. *Plant J*., **10**, 437–450.

Lindbo,J.A., Silvarosales,L., Proebsting,W.M. and Dougherty,W.G. (1993) Induction of a highly specific antiviral state in transgenic plants—implications for regulation of gene expression and virus resistance. *Plant Cell*, **5**, 1749–1759.

Lucas,W.J., Bouche-Pillon,S., Jackson,D.P., Nguyen,L., Baker,L., Ding,B. and Hake,S. (1995) Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. *Science*, **270**, 1980–1983.

Matthews,R.E.F. (1991) Transmission, movement and host range. In Matthews,R.E.F. (ed.), *Plant Virology*, 3rd Edn. Academic Press, San Diego, CA, pp. 338–378.

Meins,F.,Jr (1989) A biochemical switch model for cell-heritable variation in cytokinin requirement. In Goldberg,R. (ed.), *Molecular Basis of Plant Development*. Liss, New York, NY, pp. 13–24.

Meins,F.,Jr and Kunz,C. (1994) Gene silencing in transgenic plants: a heuristic autoregulation model. In Meyer,P. (ed.), *Current Topics in Microbiology and Immunology*. Springer-Verlag, Berlin, Germany, pp. 105–120.

Metzlaff,M., O'Dell,M., Cluster,P.D. and Flavell,R.B. (1997) RNA-mediated RNA degradation and chalcone synthase A silencing in *Petunia*. *Cell*, **88**, 845–854.

Mueller,E., Gibert,J., Brigneti,G., Davenport,G. and Baulcombe,D.C. (1995) Homology-dependent resistance: transgenic virus resistance in plants related to homology-dependent gene silencing. *Plant J*., **7**, 1001–1013.

Müller,A.J. (1983) Genetic analysis of nitrate reductase-deficient tobacco plants regenerated from mutant cells. *Mol. Gen. Genet*., **192**, 275–281.

Napoli,C., Lemieux,C. and Jorgensen,R. (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. *Plant Cell*, **2**, 279–289.

Palauqui,J.-C. and Vaucheret,H. (1995) Field trial analysis of nitrate reductase co-suppression: a comparative study of 38 combinations of transgene loci. *Plant Mol. Biol*., **29**, 149–159.

Palauqui,J.-C., Elmayan,T., Dorlhac de Borne,F., Crété,P., Charles,C. and Vaucheret,H. (1996) Frequencies, timing and spatial patterns of co-suppression of nitrate reductase and nitrite reductase in transgenic tobacco plants. *Plant Physiol*., **112**, 1447–1456.

Park,Y.-D., Papp,I., Moscone,E.A., Iglesias,V.A., Vaucheret,H., Matzke,M.A. and Matzke,A.J.M. (1996) Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. *Plant J*., **9**, 183–194.

Sijen,T., Wellink,J., Hiriart,J.-B. and van Kammen,A. (1996) RNA-mediated virus resistance: role of repeated transgenes and delineation of targeted regions. *Plant Cell*, **8**, 2277–2294.

Smith,C.J.S., Watson,C.F., Bird,C.R., Ray,J., Schuch,W. and Grierson,D. (1990) Expression of a truncated tomato polygalacturonase gene inhibits expresssion of the endogenous gene in transgenic plants. *Mol. Gen. Genet*., **224**, 477–481.

Smith,H.A., Swaney,S.L., Parks,T.D., Wernsman,E.A. and Dougherty, W.G. (1994) Transgenic plant virus resistance mediated by untranslatable sense RNAs: expression, regulation, and fate of nonessential RNAs. *Plant Cell*., **6**, 1441–1453.

Thierry,D. and Vaucheret,H. (1996) Sequence homology requirements for transcriptional silencing of 35S transgenes and post-transcriptional silencing of nitrite reductase (trans)genes by the tobacco 271 locus. *Plant Mol. Biol*., **32**, 1075–1083.

Van Blokland,R., Vandergeest,N., Mol,J.N.M. and Kooter,J.M. (1994) Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. *Plant J*., **6**, 861–877.

Van der Krol,A.R., Mur,L.A., Beld,M., Mol,J.N.M. and Stuitje,A.R. (1990) Flavonoid genes in petunia: addition of a limited number of genes copies may lead to a suppression of gene expression. *Plant Cell*, **2**, 291–299.

Vaucheret,H. (1993) Identification of a general silencer for 19S and 35S promoters in a transgenic tobacco plant: 90 bp of homology in the promoter sequences are sufficient for trans-inactivation. *C.R. Acad. Sci. Paris*, **316**, 1471–1483.

Vaucheret,H., Palauqui,J.-C., Elmayan,T. and Moffatt,B. (1995) Molecular and genetic analysis of nitrite reductase co-suppression in transgenic tobacco plants. *Mol. Gen. Genet*., **248**, 311–317.

Vaucheret,H., Nussaume,L., Palauqui,J.-C., Quilléré,I. and Elmayan,T. (1997) A transcriptionally active state is required for post-transcriptional silencing (co-suppression) of nitrate reductase host genes and transgenes. *Plant Cell*, in press.

Vernooij,B., Friedrich,L., Morse,A., Reist,R., Kolditz-Jawhar,R., Ward,E., Uknes,S., Kessmann,H. and Ryals,J. (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell*, **6**, 959–965.

Ye,F. and Signer,E.R. (1996) RIGS (repeat-induced gene silencing) in *Arabidopsis* is transcriptional and alters chromatin configuration. *Proc. Natl Acad. Sci. USA*, **93**, 10881–10886.

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